

# Isolation, Purification, and Properties of Porcine Calcitonins\*

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**ABSTRACT:** Acid extracts of hog acetone-dried thyroid powder contain three components with calcitonin activity. Two of these calcitonins (PC-1 and PC-2) have been obtained in pure form. The methionine of PC-2 was found to be in the sulfoxide form. Calcitonin PC-1 involves a single peptide chain with the empirical amino acid formula: Cys<sub>2</sub>Asx<sub>4</sub>Thr<sub>2</sub>Ser<sub>4</sub>Glx<sub>1</sub>-Pro<sub>2</sub>Gly<sub>3</sub>Ala<sub>1</sub>Val<sub>1</sub>Met<sub>1</sub>Leu<sub>3</sub>Tyr<sub>1</sub>Phe<sub>3</sub>His<sub>1</sub>Arg<sub>2</sub>Trp<sub>1</sub>. An intra-di-

sulfide bridge involving an N-terminal cysteine is present in PC-1 and PC-2. Bovine serum albumin added to assay samples of high-purity calcitonins increases the biological response by five- to sixfold. Calcitonins PC-1 and PC-2 have biological activities of approximately 270 MRC units/mg of protein. The reduced form of PC-1 gave full activity when assayed in the presence of bovine serum albumin.

**E**xtensive studies have shown that plasma calcium levels in mammals are regulated within very narrow limits. Two peptide hormones are now known to be involved in this regulation. The parathyroid hormone, released from the parathyroid gland, raises blood calcium by stimulating bone calcium resorption. Calcitonin, secreted from the ultimobranchial tissue of the thyroid, lowers the calcium level by blocking this resorption. The physiological role of these hormones has been the subject of many publications. The proceedings of three recent symposia present comprehensive reviews of the current status of the mode of action and purification of these hormones (Talmage and Belanger, 1968; Taylor, 1968; Pechet *et al.*, 1967).

One of the porcine calcitonins has now been obtained in pure form by several laboratories (Bell, 1967; Putter *et al.*, 1967; Kahnt *et al.*, 1968; Franz *et al.*, 1968; Potts *et al.*, 1968; Brewer *et al.*, 1968). The separation of three porcine calcitonins has been reported from our laboratories (Bell, 1967). The final purification of one of these porcine calcitonins (PC-1)<sup>1</sup> along with its complete structure has been reported earlier (Bell *et al.*, 1968). The results of this investigation are given in detail here and in the following paper (Barg *et al.*, 1970).

## Experimental Section

**Calcitonin Assay.** The essential features of the assay procedure used in our laboratories are as follows. Sherman rats (50–70 g in weight), fasted 18 hr prior to dosing, were injected intravenously with 0.5 ml of test sample; 1 hr later 0.05 ml (1000 units/ml) of heparin was injected intravenously followed by bleeding immediately from the superior ophthalmic vein. Plasma calcium was determined by a Technicon AutoAnalyzer adaptation of the murexide method of Williams and Moser (1956). In position-finding assays for column fractions two rats per point and a constant dilution were used.

For quantitative estimates a four-dilution, four rats per point, assay procedure was followed, comparing the unknowns with a secondary standard. Careful comparison of this standard with the Research Standard A supplied by the World Health Organization allowed these data to be expressed in the M.R.C. units.<sup>2</sup> In earlier work the samples were injected in 0.1 M sodium acetate buffer (pH 4.6). For the highly purified preparations, 0.1 M sodium acetate buffer (pH 5.0) containing 0.1% bovine serum albumin was the solvent. It was suspected that purified PC might need protective protein during assay. In earlier work from these laboratories it had been shown that 0.1% bovine serum albumin was very effective in protecting ACTH during assay (Brown *et al.*, 1955). It is shown in this publication that bovine serum albumin addition to high-purity PC preparations enhances the assay response by severalfold.

**Extraction Procedure.** Trypsin and chymotrypsin was found to inactivate crude thyroid extracts, therefore, calcitonin must contain a peptide or protein moiety which is essential for its biological action. Porcine calcitonin activity is quite stable to acid conditions with a half-life in 0.1 N HCl greater than 4 hr at 70° and about 45 min at 110°. These observations are in agreement with those reported by Tashjian and Warnock (1968). The hot acid extraction method of Gudmundsson *et al.* (1966) was used throughout. Initially 0.2 N HCl 70° (Baghdiantz *et al.*, 1964) was employed. Later this was changed to 2 N acetic acid at 70° to avoid a pH adjustment in the oxidized cellulose adsorption process described below. In all cases hog thyroid acetone-dried powder (HTAP) was the starting material.

**Selective Batch Adsorption.** Preliminary adsorption studies indicated that calcitonin was selectively picked up by acidic ion exchangers. Oxidized cellulose proved to be unusually effective when used under conditions similar to those of Astwood *et al.* (1951). Sydnor and Sayers (1952) and Shepherd *et al.* (1956). The final adsorption procedure, which gave the best compromise of purification *vs.* yield, involved the

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<sup>1</sup> Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: PC, porcine calcitonin; HTAP, hog thyroid acetone-dried powder; DNS, 1-dimethylaminonaphthalene-5-sulfonyl; AE, S-β-aminoethyl; OC, oxidized cellulose.

<sup>2</sup> MRC unit is defined by the Division of Biological Standards of the National Institute for Medical Research, Mill Hill, London, England. Their bioassay reference standard Research Standard A has an assigned potency of 0.025 MRC unit/mg.

following steps: (a) 1 kg of HTAP was suspended in 10 l. of 2 N acetic acid (70°) and stirred for 1 hr without additional heat; (b) diluted with 20 l. of cold water (8°), settled for 1–2 hr; (c) supernatant was pumped off; (d) 10 l. of cold water was added, settled, and supernatant was removed; (e) step d was repeated; (f) supernatant from c, d, and e were pooled (ca. 30 l.) and adjusted to pH 3.0 if necessary with  $\text{NH}_4\text{OH}$ ; (g) 800 g of OC (10–11% oxidized, Tennessee Eastman Co., Kingsport, Tenn.) was added. The OC had been washed once with 0.2 N HCl and twice with 10% acetic acid. The mixture was stirred at 4° for 12 hr and settled for 4 hr; (h) supernatant was removed and OC was washed twice with 6-l. portions of 10% acetic acid (4°); (i) OC was filtered and slurried with cold 0.2 N HCl (4°), the OC slurry was poured into a closed column and allowed to settle for 4–5 hr; (j) PC was eluted from the OC with 0.2 N HCl, until absorbance of eluate at 280 m $\mu$  was less than 0.1 (ca. 6 l. total); (k) Amberlite IRA 400 acetate was added to the eluate to pH 3.0 (25°) to convert the solution into the acetate form; and (l) the product PC(OC)<sup>3</sup> was recovered by lyophilization.

**Starch Gel Electrophoresis.** The urea starch gel electrophoresis method of Barg *et al.* (1965) was modified to resolve the basic substances of PC(OC). For this purpose 0.016–0.018 N HCl (pH 1.9–2.0) gave excellent resolution in 6.4 M urea starch gels. After electrophoresis at 5° using 6 V/cm for 5 hr the resolved components of the charge were visualized by nigrosin or Amido Black staining. Calcitonin activity was located in unstained sections of the gel by assaying water extracts.

**Paper Strip Chromatography.** Solvent systems using 1-butanol–H<sub>2</sub>O–acetic acid (5:3:2 or 5:4:1) were employed. After overnight development the resolved components of PC(OC) and other partially purified calcitonin preparations were located by ninhydrin staining. Location of calcitonin activity was achieved by assay of 10% acetic acid extracts of sections of unstained chromatograms.

**Butanol Precipitation.** A twofold purification of PC(OC) was readily obtained by the addition of an equal volume of 1-butanol to solutions of PC(OC) in 40% acetic acid (0.25 g of PC(OC)/ml, 25°). Inactive protein was precipitated by the 1-butanol. The organic phase which separated contained over 90% of the calcitonin. After recovery by the heptane procedure (see below) the calcitonin in the aqueous phase could be used directly in other purification steps.

**Gel Filtration.** Gel columns of either Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) or Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) proved suitable for calcitonin fractionation. These columns were developed with either 0.1 N acetic acid, or 0.1 or 1 N formic acid. Column operation conditions were essentially as described by Gudmundsson *et al.* (1966).

**Countercurrent Distribution.** The unusually high  $R_F$  of PC activity in the 1-butanol–H<sub>2</sub>O–acetic acid chromatography systems suggested the use of these solvent mixtures in counter-

current distribution studies. In all CCD procedures the volatile two-phase solvent system (1-butanol–H<sub>2</sub>O–acetic acid, 5:4:1) was employed. All extended runs were carried out at 4° to reduce calcitonin inactivation.

**Recovery Procedures.** In all cases solid products were obtained by lyophilization from aqueous solutions. In order to avoid hygroscopic preparations all final solutions were first converted, if necessary, into acetate form by treatment with Amberlite IRA 400 acetate, using the procedure of Shepherd *et al.* (1956).

Recovery of fractions from 1-butanol–H<sub>2</sub>O–acetic acid systems was accomplished by use of *n*-heptane addition, which returned the protein material into the aqueous phase where it could be quantitatively recovered in the acetate form by lyophilization. Impurities in the heptane were removed by extraction with dilute acetic acid prior to use. To assure quantitative recovery of product from the aqueous phase, the amount of *n*-heptane added should be three to four times the butanol in the system.

**Peptide and Protein Analysis.** Gel filtration columns involving relatively large charges were monitored by absorption at 280 m $\mu$ . Analysis of low-charge columns and final stages of purification required a method of greater sensitivity and general applicability to all types of peptides. For this purpose the alkaline hydrolysis–ninhydrin method of Hirs *et al.* (1956) was reduced to a micro scale. The modified procedure was used in the range of 1–20(±1)  $\mu$ g of protein. Assays were calibrated by using ribonuclease as a standard.

**Ion-Exchange Chromatography.** A modification of the pyridine–acetate volatile buffer system of Dedman *et al.* (1961) was used to develop CM-cellulose columns. CM-cellulose (Bio-Rad Cellex-CM, 0.69 mequiv/g) strongly adsorbed PC-1 and PC-2 from 0.062 M pyridine acetate buffers (pH 4.4). Some inactive material could be removed from the columns by a forerun of this buffer. Application of a linear gradient using 0.062 M, pH 4.4, and 1.86 M, pH 5.7, pyridine acetate buffers gave satisfactory fractionation. These CM-cellulose columns could be used repeatedly by removing adsorbed proteins with the pH 5.7 buffer and reequilibration with the pH 4.4 starting buffer.

**Methods of Characterization.** Ultracentrifuge studies were carried out in the Spinco Model E centrifuge. Schlieren patterns were obtained from 80-min runs at 52,640 rpm. The molecular weights were determined by the short-column equilibrium method of Yphantis (1960). The partial specific volume of 0.72 cm<sup>3</sup> g<sup>−1</sup> used in the calculations was that calculated from the amino acid analysis data. The acidic buffer system (0.05 M KCl–0.05 N HCl, pH 1.42) of Brown *et al.* (1956) was used to reduce association of the type observed with the basic peptide ACTH.

The amino acid compositions of all preparations were determined with the Technicon AutoAnalyzer (Technicon Instruments Corp., Ardsley, N. Y.) system using sulfonated polystyrene resin columns. For peptide analysis a 75-cm column procedure, using citrate buffer gradients from pH 2.75 to 6.10, as specified by Technicon, was used. For calcitonin analysis a 133-cm column developed with citrate buffers from pH 2.88 to 5.00 was employed. All samples were prepared for analysis by hydrolysis in 6 N HCl at 110° for 16–21 hr. Oxygen was removed from the hydrolysis solutions by use of a stream of prepurified nitrogen. This procedure did not entirely prevent oxidation of methionine. The total

<sup>3</sup> Sample designation in this paper reflects the purification sequence used in its preparation. The procedure and its order of use is indicated within the parentheses in the system. The procedures involved are: OC, oxidized cellulose adsorption; BP, butanol precipitation; C, G-50, Sephadex G-50 columns; BC, bottle countercurrent extractions; CCD, countercurrent distribution; CMC, and CM-cellulose chromatography.

TABLE 1: Effect of Amount of Oxycellulose on Purification and Yield of Calcitonin.

HTAP:OC	Oxycellulose Products (from 10 g of HTAP)			
	Protein (mg)	MRC units/mg	Purificn	% Act. Recovd <sup>a</sup>
2000	0.15			
200	0.84			
80	1.3	1.2	80	1.1
40	2.5	1.0	66	1.9
20	5.4	1.1	73	4
10	13.1	2.1	140	19
5	23.7	1.4	94	22

<sup>a</sup> Assay reliability ~ (0.5-2.0) × value shown.

methionine was considered to be the sum of methionine, methionine sulfone, and methionine sulfoxide. Cysteine was determined on separate samples either oxidized with performic to cysteic acid by the method of Moore (1963) or as AE-cysteine after reduction and aminoethylation by the method of Raftery and Cole (1966). Tryptophan was determined on unhydrolyzed samples by the colorimetric method of Spies and Chambers (1948) or after release by a trypsin-carboxypeptidase B digestion as described by Barg *et al.* (1970).

Free thiol determinations were made using the *p*-mercuribenzoate method of Boyer (1954). These studies were carried out in 0.33 M sodium acetate buffer (pH 4.6).

End groups were studied using enzyme digestions with DFP-treated carboxypeptidases A and B, and leucine aminopeptidase obtained from Worthington Biochemical Co., Freehold, N. J., and micrococcal aminopeptidase (Rohm and Haas, Darmstadt, Germany) obtained from Henley and Co., New York, N. Y. Calcitonin digestions with these enzymes were carried out at 37° in 0.2 M NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.0) using substrate to enzyme ratios of 10 to 100. The leucine aminopeptidase was first activated by incubation at 37° for 90 min in 0.025 M Tris buffer (pH 8.4) which contained 0.001 M MnCl<sub>2</sub> (*ca.* 1 mg of enzyme protein/ml). Terminal amino studies were also investigated by the dansylation method of Gray and Hartley (1963). The N-terminal DNS-amino acid released by acid hydrolysis was identified on polyamide thin-layer plates (Gallard-Schlesinger Chemical Manufacturing Co., Long Island, N. Y.) by the method of Woods and Wang (1967).

## Results and Discussion

**Oxycellulose Adsorption of Calcitonin.** Range finding experiments in which the amount of OC was varied gave the data shown in Table I. All products obtained in this experiment were heterogeneous when studied in starch gel electrophoresis. In order to improve activity recoveries, an HTAP:OC ratio of 1.25 was selected. The average activity yield obtained by the final procedure was about 25% with a purification, on a protein basis, of 40-80-fold. Repeat adsorptions with OC recovered additional hormone but of lower activity. Also, the 0.2 N HCl elution of activity from the

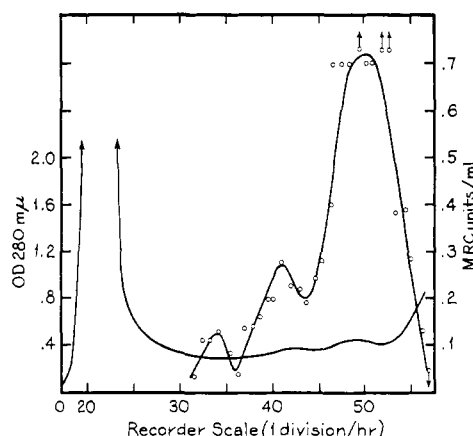


FIGURE 1: Sephadex G-50 column fractionation of PC(OC). G-50 (Superfine) column (3.4 × 182 cm). Charge: 2.0 g of PC(OC). Developed with 1.0 N formic acid, upward flow 27.5 ml/hr, 4°. —OD<sub>280 mμ</sub>, (○—○) activity. In the critical area (40-48 on the recorder chart) two bioassay levels, both in the response range, were used.

OC was never complete. This loss was minimized by the reuse of the OC after two washes with 10% acetic acid.

**Starch Gel Electrophoresis.** Studies of PC(OC) preparations in the pH 2.0, 6.4 M urea starch gels showed about 20 sharp bands which had moved toward the cathode. Calcitonin activity was not associated with any particular one of these bands. The calcitonin activity had migrated approximately 5 cm toward the cathode.

**Paper Chromatography.** Paper chromatography studies using the 1-butanol-H<sub>2</sub>O-acetic acid systems resolved PC(OC) material into several bands. Bioassay showed the calcitonin moved ahead of most of the ninhydrin-positive material with an *R<sub>F</sub>* of about 0.6 in both systems.

**Gel Filtration.** Gel columns using Sephadex G-50 superfine proved useful as an intermediate stage of calcitonin purification. Long columns, such as those shown in Figure 1, suggested the presence of three calcitonins in PC(OC). The active fractions of this column were recovered and rerun on a smaller G-50 column. Activity did not center on the protein peak in these columns, demonstrating the limitation of this gel column as a final stage purification procedure for PC. The 1 N formic acid was selected initially to maintain the pH below the optimum of the proteolytic enzymes of the hog thyroid described by Kress *et al.* (1966). Low recoveries of biological activity for the 1 N formic acid columns (no bovine serum albumin in assay) led to the use of weaker acid conditions which gave satisfactory yields when bovine serum albumin was added to the assay samples. Preparative Sephadex G-50 columns (10 × 150 cm) were charged with 20 g of PC(OC, BP) and developed at 4° with 0.1 N acetic acid. The active pool gave an average recovery of about 80% with a purification of 5-10-fold (bovine serum albumin in assay).

**Countercurrent Distribution.** The unusually high *R<sub>F</sub>* of PC in the paper strip studies mentioned above suggested the application of countercurrent distribution as a useful purification procedure. A preliminary distribution of PC(OC) material in the system 1-butanol *vs.* 20% acetic acid, run 20 transfers at 25°, showed that the activity was ahead of the bulk of the protein of the charge. Continuation of this run

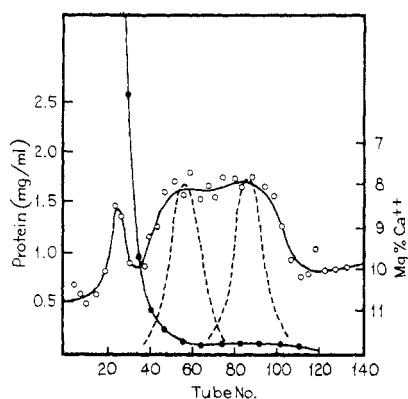


FIGURE 2: Countercurrent distribution of calcitonin. PC(OC, G-50) (0.5 g) charged in two tubes of a 250-tube machine (11 ml/phase), 4°;  $n = 225$ . (●—●) Protein analysis, (○—○) biological activity, and (----) theoretical curves.

to 1000 transfers led to inactivation. In order to reduce this rate of inactivation, all subsequent countercurrent distributions in automatic machines were carried out at 4°.

The first CCD experiment carried out at 4° gave the results shown in Figure 2. As expected, the bulk of the calcitonin activity moved ahead of the protein. From consideration of the assay data and theoretical curves, three active species are indicated. These were designated as PC-1 (tubes 80–100), PC-2 (tubes 50–70), and PC-3 (tubes 20–30). At least a 50-fold purification of PC-1 and PC-2 was realized by this procedure. Extending the experiment of Figure 2 to 835 transfers completely separated the three calcitonin activities. The PC-3 activity was still associated with the slow-moving protein.

The data of Figure 2 made it clear that large amounts of HTAP must be processed to obtain the minimum amount of the pure hormones needed for structural studies. As is usually the case with countercurrent procedures, emulsion problems become serious if more concentrated solutions are used. In order to overcome this difficulty a bottle distribution experiment designed to remove the bulk of the protein impurities was developed. Distribution coefficient data from

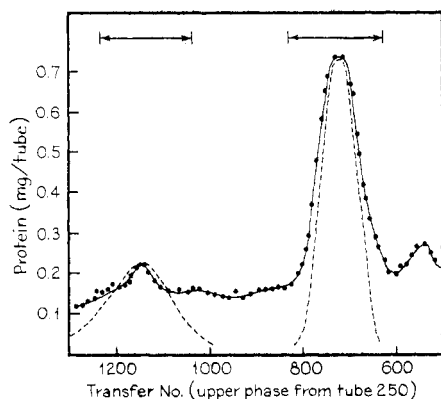


FIGURE 3: Countercurrent distribution of calcitonin. PC(OC, BP, G-50, BC) (0.506 g) charged in 11 tubes (11 ml/phase), 4°. (—) Protein analysis, (----) theoretical curve, and (↔) activity greater than 12 MRC units/tube.

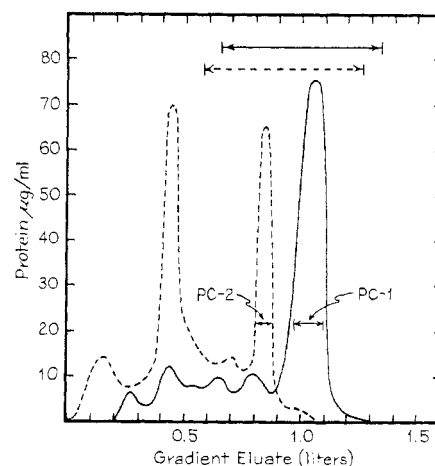


FIGURE 4: Chromatography of PC-1 and PC-2 on CM-cellulose column. Column details: size,  $2.5 \times 120$  cm; equilibrated and charged in 0.062 M pyridine acetate buffer, pH 4.4 (5 ml of pyridine and 8.2 ml of acetic acid per l.); forerun, 2.2 l., pH 4.4 buffer; development by linear gradient 1 l., pH 4.4, buffer, 1 l., pH 5.7, buffer (150 ml of pyridine and 35 ml of acetic acid/l.); flow rate, ca. 35 ml/hr. Column run 1: PC-1 (200 mg) from CCD charged in 20 ml, pH 4.4 buffer. (—) Protein, (↔) activity greater than 1.2 MRC units/ml. Column run 2: PC-2 (160 mg) from CCD charged in 32 ml, pH 4.4, buffer. (----) Protein analysis, (↔) activity greater than 1.2 MRC units/ml.

Figure 2 made it possible to estimate that a six-bottle 26-transfer experiment should recover 80% of PC-1 and 40% of PC-2 in the pool of the top phases collected from bottle 6. This pool should contain no more than 1–2% of PC-3 and protein impurities. A typical application of this procedure is given below.

A charge of 4.29 g of PC(OC, G-50) was carried through a bottle experiment using 100 ml of each phase of the 1-butanol- $H_2O$ -acetic acid (5:4:1, v/v) system. The lower phase was placed in all six bottles and the charge was introduced in the upper phase of the first bottle. The material recovered [PC(OC, G-50, BC)] from 20 top phases collected from bottle 6 after 26 transfers amounted to 0.263 g and had the expected increase in specific activity.

Subsequent isolations of PC-1 and PC-2 by the CCD procedure were carried out using PC(OC, BP, G-50, BC)-type material. Larger amounts of this material could be charged in the CCD machine with a minimum of emulsion problems. Both PC-1 and PC-2 were recovered from upper-phase fractions collected from tube 250 of the machine. A typical experiment involving a charge of 0.506 g [equivalent to 8.806 g of PC(OC, BP, G-50)] gave the data shown in Figure 3. It should be pointed out that the PC-1 to PC-2 ratio has been increased at the expense of PC-2 by the bottle countercurrent step. The calcitonin activity was found to center on the two peaks of the protein analysis. Comparison of the protein analysis, theoretical curves, amino acid analysis, ultracentrifuge studies, and fingerprint studies of trypsin and chymotrypsin digests showed that the PC-1 peak (650–800 of Figure 3) was nearly pure. By the same criteria, the PC-2 peak (1050–1250) was less homogeneous.

**Ion-Exchange Chromatography.** In order to take advantage of the basic nature of calcitonin, acidic ion exchangers were investigated. Sulfonated resins adsorbed calcitonin so tightly

TABLE II: Amino Acid Composition of Calcitonins.

Amino Acid	PC-1			PC-2		
	Mole %	Mole Ratio His = 1.0	Residues/Mole	Mole %	Mole Ratio His = 1.0	Residues/Mole
Asp	11.78	3.90	4	13.29	4.07	4
Thr	6.10	2.01	2	6.26	1.92	2
Ser	13.46	4.44	4	10.18	3.10	3?
Glu	2.96	0.98	1	3.32	1.02	1
Pro	6.25	2.12	2	5.69	1.75	2
Gly	9.44	3.11	3	9.29	2.85	3
Ala	3.37	1.11	1	4.36	1.34	1
Val	3.15	1.04	1	3.50	1.07	1
Cys	6.90 <sup>a</sup>	2.28	2	6.85 <sup>c</sup>	2.10	2
Met	2.73	0.90	1	2.14	0.66	1
Ile				0.36	0.10	
Leu	8.98	2.96	3	10.06	3.07	3
Tyr	3.15	1.04	1	2.70	0.83	1
Phe	9.06	2.99	3	9.79	3.00	3
Lys				0.39	0.12	
His	3.03	1.00	1	3.26	1.00	1
Arg	6.48	2.14	2	6.56	2.01	2
Trp	3.15 <sup>b</sup>	1.04	1	2.02 <sup>b</sup>	0.62	1

<sup>a</sup> As CysSO<sub>3</sub>H on separate sample. <sup>b</sup> Determined on a separate sample following trypsin, carboxypeptidase B digestion. <sup>c</sup> As AE-Cys on separate sample.

that they did not prove useful. Carboxy resin columns of the type which were used successfully in ACTH purification by Dixon and Stack-Dunne (1955) and Farmer and Morris (1956) fractionated material but led to calcitonin inactivation. CM-cellulose columns proved useful for further purification of calcitonin preparations from countercurrent distributions. The data for the purification of PC-1 and PC-2 are shown in Figure 4.

Assay levels in the experiments of Figure 4 were too high to give good definition of the peaks. The PC-1 and PC-2 protein peaks were pooled as indicated and were used for final characterization and structural studies. The peak positions from the two experiments indicate that this CM-cellulose column may have the resolving power to separate PC-1 and PC-2. Brewer *et al.* (1968) obtained a partial separation of two active porcine calcitonins with a similar column developed with ammonium acetate buffers. A repeat countercurrent run on the PC-1 of Figure 4 gave a single PC-1 peak by protein analysis which was in excellent agreement with a theoretical curve both as to shape and position. However, bioassay of this CCD showed small amounts of activity at the position corresponding to PC-2. Since the CCD procedure clearly separated PC-1 activity from PC-2 initially, a small amount of PC-1 must have been converted into PC-2 either in the CCD recovery process or by the CM-cellulose column and its recovery procedure.

**Characterization of Porcine Calcitonins.** End-group studies with leucine aminopeptidase, micrococcal aminopeptidase, carboxypeptidases A and B, all failed to release terminal amino acids from either PC-1 or PC-2. Amino-terminal cysteine was identified in both PC-1 and PC-2 by the dansylation method.

Ultracentrifugal studies of PC-1 and PC-2 gave schlieren patterns which closely fit single Gaussian distributions throughout the experimental runs. The  $s_{20,w}^0$  values obtained were PC-1, 0.81 S, and PC-2, 0.71 S. The molecular weights determined by the short-column procedures were 3740 for PC-1 and 3420 for PC-2. These determinations are in reasonable agreement with the minimum molecular weight calculated from the amino acid analysis shown in Table II. No free thiol groups were present in PC-1 therefore the two cysteines are present in an intra-disulfide form.

The CM-cellulose column procedure removes the last traces of lysine and isoleucine from PC-1. The PC-2 prepared by this method still contained trace amounts of these amino acids. The lower serine content of PC-2 is not considered real. Fingerprint comparisons with PC-1 by the methods described by Barg *et al.* (1970) did not show any peptides missing a serine residue. Total enzyme digestions of PC-1 and PC-2 using a papain-micrococcal aminopeptidase procedure similar to that described by Brewer *et al.* (1968) released 1 mole of methionine from PC-1. Nearly all of the methionine of PC-2 was released as the methionine sulfoxide by this method. These data suggest that PC-2 is the methionine sulfoxide form of PC-1. Further, application of the DNS-Edman stepwise degradation procedure as described by Barg *et al.* (1970), gave a sequence for PC-2 which was identical with that of PC-1 for the first 16 amino acids from the N terminus.

Biological assay experiments with high-purity calcitonin preparations demonstrated the importance of protective protein in the final solutions being assayed. The assay data for PC-1 at the countercurrent stage of purity are shown in Figure 5. In other assays it was shown that the bovine serum

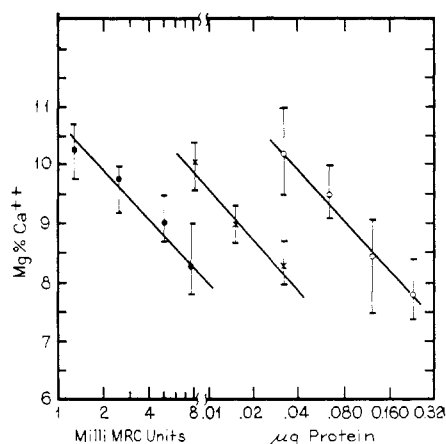


FIGURE 5: Biological assays of MRC standard A and PC-1 with and without bovine serum albumin. Four rats per point. (—) Range of results, (—●—) MRC standard with 0.1% bovine serum albumin, (—×—) PC-1 with 0.1% bovine serum albumin, and (—○—) PC-1 without bovine serum albumin.

albumin did not have any calcitonin activity alone, further, it did not increase the response of the crude MRC standard. The bovine serum albumin enhancement of the high-purity PC-1 is approximately fivefold. It has been suggested by Parsons (Taylor, 1968) that at pH 3.0, bovine serum albumin prevents adsorption of calcitonin on the surface of the vessels used in the preparation of assay samples. The pH 5.0 data of Figure 5 are not compatible with this thesis. No significant change in slope of the dose response curve was ever observed for high-purity samples assayed with or without bovine serum albumin from that of crude samples with or without bovine serum albumin added. Assay comparisons of PC-1 (CMC) and PC-2 (CMC) with the PC-1 (CCD) preparation of Figure 5 indicated that these samples were equally active (approximately 270 MRC units/mg of protein).

Preliminary experiments indicate that the presence of the intra-disulfide bridge in PC-1 is not an absolute requirement for biological activity. After treatment with 2.9 M 2-mercaptoethanol at pH 5.9 for 2 hr, 25°, the reduced PC-1 solution was diluted with 0.2% bovine serum albumin in 0.02 M sodium acetate buffer (pH 5.9) for assay. These reduced samples were just as active as the exact duplicates which had not been treated with the 2-mercaptoethanol.

#### Acknowledgments

The authors thank E. Heyder, E. Lindemann, and H. Falk for their invaluable assistance in the bioassay studies; M. C. Davies and R. Hulbert for amino acid analyses, M. E. Englert molecular weight studies as well as H. Seigrist and C. Pidacks for their collaboration during the purification work. We also wish to express particular appreciation to Dr. I. Ringler for his interest and encouragement throughout the course of these studies.

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